



Brilliant QPCR Master Mix

Instruction Manual

Catalog #600549 (single kit)

#929549 (10-pack kit)

Revision C

Research Use Only. Not for Use in Diagnostic Procedures.

600549-12



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Brilliant QPCR Master Mix

CONTENTS

Materials Provided	1
Storage Conditions	1
Additional Materials Required	1
Notices to Purchaser	1
Introduction	2
Molecular Beacons	3
TaqMan® Probes (Hydrolysis Probes).....	3
Endpoint vs. Real-Time Measurements.....	3
Fluorescence Monitoring in Real-Time.....	5
Preprotocol Considerations	7
Magnesium Chloride Concentration.....	7
Probe Design	7
Fluorescence Detection	7
Optimal Concentrations for Experimental Probes and Primers.....	7
Reference Dye	8
Data Acquisition with a Spectrofluorometric Thermal Cycler.....	8
Preventing Template Cross-Contamination.....	9
Multiplex PCR.....	9
Protocol	11
Preparing the Reactions	11
PCR Cycling Programs.....	12
Troubleshooting: Molecular Beacons	13
Troubleshooting: TaqMan® Probes	14
References	15
Endnotes	15
MSDS Information	15
Quick-Reference Protocol	17

Brilliant QPCR Master Mix

MATERIALS PROVIDED

Catalog #600549 (single kit), #929549 (10-pack kit)

Materials provided (per kit)	Quantity ^{a,b}
2× Brilliant QPCR Master Mix	2 × 2.5 ml
Reference dye ^c , 1 mM	100 µl

^a Sufficient PCR reagents are provided for four hundred, 25-µl reactions.

^b Quantities listed are for a single kit. For 10-pack kits, each item is provided at 10 times the listed quantity.

^c The reference dye is light sensitive and should be kept away from light whenever possible.

STORAGE CONDITIONS

All Components: Upon receipt, store all components at –20°C. Store the 2× master mix at 4°C after thawing. Once thawed, full activity is guaranteed for 6 months.

Note *The reference dye is light sensitive and should be kept away from light whenever possible.*

ADDITIONAL MATERIALS REQUIRED

Spectrofluorometric thermal cycler
Nuclease-free PCR-grade water

NOTICES TO PURCHASER

Notice to Purchaser: Limited License

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Revision C

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INTRODUCTION

Quantitative PCR is a powerful tool for gene expression analysis. Many fluorescent chemistries are used to detect and quantitate gene transcripts. The use of fluorescent probe technologies reduces the risk of sample contamination while maintaining convenience, speed, and high throughput screening capabilities. The Brilliant QPCR Master Mix is a single-tube reagent ideal for most high-throughput QPCR applications. Brilliant kits support quantitative amplification and detection with multiplex capability and show consistent high performance with various fluorescent detection systems, including molecular beacons and TaqMan® probes. The Brilliant QPCR master mix includes the components necessary to carry out PCR amplifications*, and has been successfully used to amplify and detect a variety of DNA targets, including genomic DNA, plasmid DNA, and cDNA.

The Brilliant QPCR master mix has been optimized for maximum performance on Stratagene Mx3000P, Mx3005P, and Mx4000 instruments, as well as on the ABI PRISM® 7700 instrument. In addition, excellent results have been observed using most other QPCR platforms.

The Brilliant QPCR master mix includes SureStart *Taq* DNA polymerase, a modified version of *Taq2000* DNA polymerase with hot start capability. SureStart *Taq* DNA polymerase improves PCR amplification reactions by decreasing background from non-specific amplification and increasing amplification of desired products. Using SureStart *Taq*, hot start is easily incorporated into PCR protocols already optimized with *Taq* DNA polymerase, with little modification of cycling parameters or reaction conditions.

A passive reference dye (an optional reaction component) is provided in a separate tube; providing this reagent separately allows the user to control the final dye concentration, increasing the flexibility of the reagents for use with different platforms.

* Primers and template are not included.

Molecular Beacons

Molecular beacons are hairpin-shaped fluorescent hybridization probes that can be used to monitor the accumulation of specific product during or after PCR.¹⁻⁵ Molecular beacons have a fluorophore and a quencher molecule at opposite ends of an oligonucleotide. The ends of the oligonucleotide are designed to be complementary to each other. When the unhybridized probe is in solution, it adopts a hairpin structure that brings the fluorophore and quencher sufficiently close to each other to allow efficient quenching of the fluorophore. If, however, the molecular beacon is bound to its complementary target, the fluorophore and quencher are far enough apart that the fluorophore cannot be quenched and the molecular beacon fluoresces (see Figure 1). As PCR proceeds, product accumulates and the molecular beacon fluoresces at a wavelength characteristic of the particular fluorophore used. The amount of fluorescence at any given cycle depends on the amount of specific product present at that time.

TaqMan® Probes (Hydrolysis Probes)

TaqMan probes are linear.^{6, 7} The fluorophore is usually at the 5' end of the probe, and the quencher is either internal or is at the 3' end. As long as the probe is intact, regardless of whether it is hybridized with the target or free in solution, no fluorescence is observed from the fluorophore. During the combined annealing–extension step of PCR, the primers and the TaqMan probe hybridize with the target (see Figure 2). The DNA polymerase displaces the TaqMan probe by 3 or 4 nucleotides, and the 5'-nuclease activity of the DNA polymerase separates the fluorophore from the quencher. Because of this mechanism of action, these probes are also referred to as hydrolysis probes. Fluorescence can be detected during each PCR cycle, and fluorescence accumulates during the course of PCR.

Endpoint vs. Real-Time Measurements

The probes can be used in a variety of PCR applications, including infectious agent detection, genotyping, detection of single nucleotide polymorphism (SNP) variants, allelic discrimination, and quantitative gene expression analysis. The fluorescence of the probe can be monitored either when cycling is complete (endpoint analysis) or as the reaction is occurring (real-time analysis). For endpoint analysis, PCR reactions can be run on any thermal cycler and can then be analyzed with a fluorescence plate reader that has been designed to accommodate PCR tubes and that is optimized for the detection of PCR reactions that include fluorescent probes. Real-time experiments are typically performed on an instrument capable of detecting fluorescence from samples during each cycle of a PCR protocol.

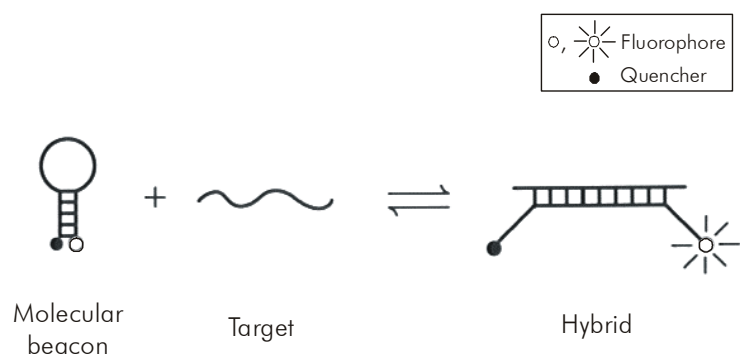


FIGURE 1 The molecular beacon binds to a complementary target and fluoresces.

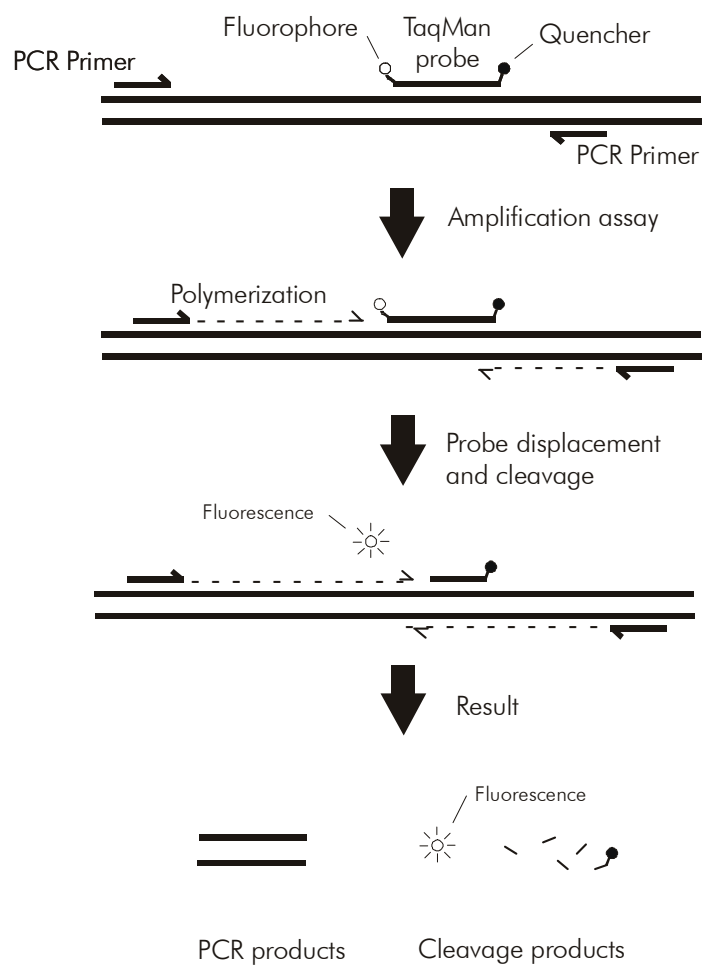


FIGURE 2 TaqMan probe fluoresces when the 5'-nuclease activity of the DNA polymerase separates the fluorophore from quencher.

Fluorescence Monitoring in Real-Time

When fluorescence signal from a PCR reaction is monitored in real-time, the results can be displayed as an amplification plot (see Figure 3), which reflects the change in fluorescence during cycling. This information can be used during PCR experiments to quantitate initial copy number. Quantitative assessments based on endpoint fluorescence values (a single reading taken at the end of the PCR reaction) are inherently inaccurate because endpoint values can be greatly influenced by limiting reagents and small differences in reaction components or cycling parameters. Studies have shown that initial copy number can be quantitated during real-time PCR analysis based on threshold cycle (Ct).⁶ Ct is defined as the cycle at which fluorescence is determined to be statistically significant above background. The threshold cycle is inversely proportional to the log of the initial copy number.⁶ The more template that is initially present, the fewer the number of cycles it takes to reach the point where the fluorescence signal is detectable above background. Quantitative information based on threshold cycle is more accurate than information based on endpoint determinations because threshold cycle is based on measurements taken during the exponential phase of PCR amplification when PCR efficiency is not yet influenced by limiting reagents, small differences in reaction components, or cycling conditions.

Figure 3 shows Mx4000 instrument amplification curve plots and Ct determination using TaqMan probes for either the TNF- α gene (top plot) or the control “housekeeping” gene GAPDH (bottom plot) using the Brilliant QPCR master mix. In this experiment, amplification of the two genes was performed either in separate reactions or in a duplex reaction at three different target DNA concentrations (10-fold increments). The data in Figure 3 demonstrates that reactions performed using either a single probe or duplexed probes in a single reaction yield very similar Ct values.

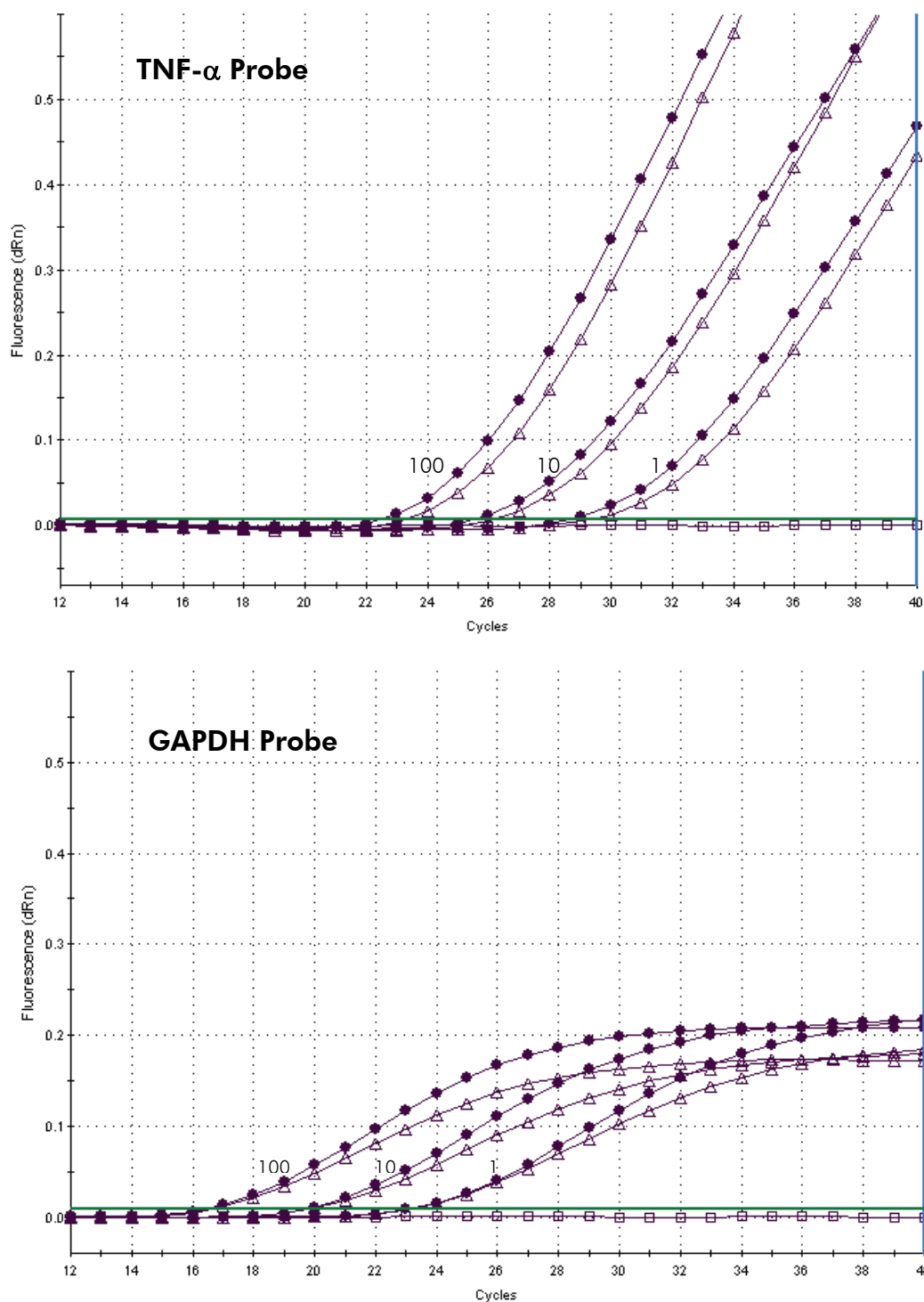


Figure 3 Mx4000 multiplex quantitative PCR instrument amplification plots using TaqMan probes for TNF- α (top panel) or GAPDH (bottom panel). The amount of template added per reaction (in ng) is indicated to the left of each set of curves. Amplification plots for single probe reactions are shown as filled circles (●), and for duplexed probes are shown as open triangles (Δ). The fluorescence value used to determine Ct (the threshold line) is shown as a solid line. Although fluorescence data was collected throughout the amplification procedure, for illustrative purposes, the data shown is limited to that collected after cycle 12.

PREPROTOCOL CONSIDERATIONS

Magnesium Chloride Concentration

Magnesium chloride concentration affects the specificity of the PCR primers and probe hybridization. The Brilliant QPCR master mix contains MgCl_2 at a concentration of 5.5 mM (in the 1× solution), which is suitable for most targets.

Probe Design

Probes should have a melting temperature that is 7–10°C higher than the annealing temperature of the primers. For additional considerations in designing TaqMan probes, refer to Primer Express® oligo design software from Applied Biosystems.

Resuspend lyophilized custom molecular beacon or TaqMan probes in buffer containing 5 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA (low TE buffer).

Fluorescence Detection

Fluorescence may be detected either in real-time or at the endpoint of cycling using a real-time spectrofluorometric thermal cycler. For endpoint analysis, PCR reactions can be run on any thermal cycler and can then be analyzed with a fluorescence plate reader that has been designed to accommodate PCR tubes and that is optimized for the detection of PCR reactions that include fluorescent probes.

If using a fluorescence reader, it is recommended that readings be taken both before and after PCR for comparison.

Optimal Concentrations for Experimental Probes and Primers

Probes

The optimal concentration of the experimental probe should be determined empirically. The optimal concentration is the lowest concentration that results in the lowest C_t and an adequate fluorescence for a given target concentration.

A) Molecular Beacons

The molecular beacon concentration can be optimized by varying the final concentration from 200 to 500 nM in increments of 100 nM.

B) TaqMan® Probes

The TaqMan probe concentration can be optimized by varying the final concentration from 100 to 500 nM in increments of 100 nM.

PCR Primers

The optimal concentration of the upstream and downstream PCR primers should also be determined empirically. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. The primer concentration for use with molecular beacons can be optimized by varying the concentration from 200 to 600 nM. The primer concentration for use with TaqMan probes can be optimized by varying the concentration from 50 to 600 nM. The best concentrations of the upstream and downstream primers are not always of equal molarity.

Reference Dye

A passive reference dye is included in this kit and may be added to compensate for non-PCR related variations in fluorescence. Fluorescence from the passive reference dye does not change during the course of the PCR reaction but provides a stable baseline to which samples are normalized. In this way, the reference dye compensates for changes in fluorescence between wells caused by slight volume differences in reaction tubes. The excitation and emission wavelengths of the reference dye are 584 nm and 612 nm, respectively. Although addition of the reference dye is optional when using the Mx4000, Mx3000P or Mx3005P system, with other instruments (including the ABI 7900HT and ABI PRISM 7700) the use of the reference dye may be required for optimal results.

Reference Dye Dilution Recommendations

Prepare **fresh*** dilutions of the reference dye prior to setting up the reactions, and **keep all tubes containing the reference dye protected from light as much as possible**. Make initial dilutions of the reference dye using nuclease-free PCR-grade H₂O. If using the Stratagene Mx3000P, Mx3005P, or Mx4000 instrument, use the reference dye at a final concentration of 30 nM. If using the ABI PRISM 7700 instrument, use the reference dye at a final concentration of 300 nM. For other instruments, use the following guidelines for passive reference dye optimization. For instruments that allow excitation at ~584 nm (including most tungsten/halogen lamp-based instruments and instruments equipped with a ~584 nm LED), begin optimization using the reference dye at a final concentration of 30 nM. For instruments that do not allow excitation near 584 nm, (including most laser-based instruments) begin optimization using the reference dye at a final concentration of 300 nM.

Data Acquisition with a Spectrofluorometric Thermal Cycler

Acquisition of real-time data generated by fluorogenic probes should be performed as recommended by the instrument's manufacturer. Data should be collected at the annealing step of each cycle (3-step cycling protocol) or the annealing/extension step (2-step cycling protocol).

* The diluted reference dye, if stored in a light-protected tube at 4°C, can be used within the day for setting up additional assays.

Preventing Template Cross-Contamination

Take precautions to minimize the potential for carryover of nucleic acids from one experiment to the next. Use separate work areas and pipettors for pre- and post-amplification steps. Use positive displacement pipets or aerosol-resistant pipet tips.

dUTP is used instead of dTTP in the Brilliant QPCR master mix. When dUTP replaces dTTP in PCR amplification, treatment with UNG (Uracil-N-glycosylase, not provided in this kit) can prevent the subsequent reamplification of dU-containing PCR products. UNG acts on single- and double-stranded dU-containing DNA by hydrolysis of uracil-glycosidic bonds at dU-containing DNA sites. When this strategy is put to use, carry-over contamination will be eliminated while template DNA (DNA containing T) will be left intact.

Multiplex PCR

Multiplex PCR is the amplification of more than one target in a single polymerase chain reaction.⁸ Multiplex PCR in which two or more sequences are amplified simultaneously can often be performed using the conditions for amplification of a single sequence.⁹ The Brilliant QPCR master mix has been successfully used to amplify two targets in a multiplex reaction without reoptimizing the concentrations of DNA polymerase or dNTPs.

In a typical multiplex PCR, one primer pair primes the amplification of the target of interest and another primer pair primes the amplification of an endogenous control. For accurate analysis, it is important to minimize competition between concurrent amplifications for common reagents. To minimize competition, the limiting primer concentrations need to be determined for the more abundant target.¹⁰ Consideration should also be given to optimization of the other reaction components. The number of fluorophores in each tube can influence the analysis. The following guidelines are useful for multiplex PCR.

PCR Primer Considerations for Multiplex PCR

- ♦ Design primer pairs with similar annealing temperatures for all targets to be amplified.
- ♦ To avoid duplex formation, analyze the sequences of primers and probes with primer analysis software.
- ♦ The limiting primer concentrations are the primer concentrations that result in the lowest fluorescence intensity without affecting the Ct. If the relative abundance of the two targets to be amplified is known, determine the limiting primer concentrations for the most abundant target. If the relative abundance of the two targets is unknown, determine the limiting primer concentrations for both targets. The limiting primer concentrations are determined by running serial dilutions of those forward and reverse primer concentrations optimized for one-probe detection systems, but maintaining a constant target concentration. A range of primer concentrations of 20–200 nM is recommended. Running duplicates or triplicates of each combination of primer concentrations within the matrix is also recommended.¹⁰

Probe Considerations for Multiplex PCR

A) Molecular Beacons

- ♦ Label each molecular beacon with a spectrally distinct fluorophore.¹¹ The use of a dark quencher may enhance the quality of multiplex PCR results.
- ♦ Design molecular beacons for different targets to have different stem sequences.

B) TaqMan® Probes

Label each TaqMan probe with a spectrally distinct fluorophore. The use of a dark quencher may enhance the quality of multiplex PCR results.

PROTOCOL

Preparing the Reactions

Notes *Once the tube containing the Brilliant QPCR master mix is thawed, store it on ice while setting up the reactions. Following initial thawing of the master mix, store the unused portion at 4°C. Multiple freeze-thaw cycles should be avoided.*

It is prudent to set up a no-template control reaction to screen for contamination of reagents or false amplification.

Consider performing an endogenous control reaction to distinguish true negative results from PCR inhibition or failure. For information on the use and production of endogenous controls for QPCR, see Reference.[Bustin, 2000 #594]

1. If the reference dye will be included in the reaction, (optional), dilute the dye solution provided **1:500 (for the Mx3000P, Mx3005P, and Mx4000 instruments)** or **1:50 (for the ABI PRISM 7700 instrument)** using nuclease-free PCR-grade H₂O. For other instruments, use the guidelines in the *Reference Dye* section under *Preprotocol Considerations*. When used according to the protocol below, this will result in a final reference dye concentration of 30 nM for the Mx3000P, Mx3005P, and Mx4000 instruments and 300 nM for the ABI PRISM 7700 instrument. **Keep all solutions containing the reference dye protected from light.**

Note *If using a system other than the Mx4000, Mx3000P or Mx3005P instruments, the use of the reference dye may be required for optimal results.*

2. Prepare the experimental reaction by adding the following components *in order*:

Experimental Reaction

Nuclease-free PCR-grade H₂O to adjust the final volume to 25 µl (including experimental DNA)

12.5 µl of 2× master mix

x µl of experimental probe (optimized concentration)

x µl of upstream primer (optimized concentration)

x µl of downstream primer (optimized concentration)

0.375 µl of the **diluted** reference dye (optional)

Note: *A total reaction volume of 50 µl may also be used.*

3. Gently mix the reactions without creating bubbles (do not vortex).
4. Add *x* µl of experimental gDNA, cDNA, or plasmid DNA to each experimental reaction.

5. Gently mix the reactions without creating bubbles (do not vortex).

Note *Bubbles interfere with fluorescence detection.*

PCR Cycling Programs

6. Centrifuge the reactions briefly.
7. Place the reactions in the instrument and run the appropriate PCR program below. Generally, TaqMan reactions utilize the 2-step program, and molecular beacons reactions utilize the 3-step program. These amplification protocols are recommended initially, but optimization may be necessary for some primer/template systems.

Two-step Cycling Protocol

Cycles	Duration of cycle	Temperature
1	10 minutes ^a	95°C
40	15-30 seconds	95°C
	1.0 minute ^b	55–60°C ^c

^a Initial 10 minute incubation is required to fully activate the DNA polymerase.

^b Set the temperature cycler to detect and report fluorescence during the annealing/extension step of each cycle.

^c Choose an appropriate annealing temperature for the primer set used.

Three-step Cycling Protocol

Cycles	Duration of cycle	Temperature
1	10 minutes ^a	95°C
40	30 seconds	95°C
	1.0 minute ^b	55–60°C ^c
	30 seconds	72°C

^a Initial 10 minute incubation is required to fully activate the DNA polymerase.

^b Set the temperature cycler to detect and report fluorescence during the annealing/extension step of each cycle.

^c Choose an appropriate annealing temperature for the primer set used.

TROUBLESHOOTING: MOLECULAR BEACONS

Observation	Suggestion
There is a low increase in fluorescence with cycling or none at all	The molecular beacon is not binding to the target efficiently because the loop portion is not completely complementary. Perform a melting curve analysis to determine if the probe binds to a perfectly complementary target.
	The molecular beacon is not binding to the target efficiently because the annealing temperature is too high. Perform a melting curve analysis to determine the optimal annealing temperature.
	The molecular beacon is not binding to the target efficiently because the PCR product is too long. Design the primers so that the PCR product is <150 bp in length.
	Design the molecular beacon with a stem that is compatible with 5.5 mM MgCl ₂ .
	For multiplex PCR, the MgCl ₂ concentration may be increased, if desired, by adding a small amount of concentrated MgCl ₂ (not provided in this kit) to the 1 × experimental reaction at the time of set up.
	The molecular beacon has a nonfunctioning fluorophore. Verify that the fluorophore functions by detecting an increase in fluorescence in the denaturation step of thermal cycling or at high temperatures in a melting curve analysis. If there is no increase in fluorescence, resynthesize the molecular beacon.
	Resynthesize the molecular beacon using a different fluorophore.
	Redesign the molecular beacon.
	The reaction is not optimized and no or insufficient product is formed. Verify formation of enough specific product by gel electrophoresis.
	For multiplex PCR of more than two targets, the master mix may need to be supplemented with additional polymerase and dNTPs (not provided in this kit).
There is an increase in fluorescence in control reactions without template	The reaction has been contaminated. Follow the procedures outlined in reference 12 to minimize contamination.
	Perform decontamination during amplification by including uracil-N-glycosylase (UNG) in the PCR reaction mix. See <i>Preventing Template Cross Contamination</i> in <i>Preprotocol Considerations</i> .
Ct reported for the no-target control (NTC) sample in experimental report is less than the total number of cycles but the curve on the amplification plot is horizontal	Variation in fluorescence intensity. Review the amplification plot and, if appropriate, adjust the threshold accordingly.

TROUBLESHOOTING: TAQMAN® PROBES

Observation	Suggestion
There is a low increase in fluorescence with cycling or none at all	The probe is not binding to the target efficiently because the annealing temperature is too high. Verify the calculated melting temperature using appropriate software.
	The probe is not binding to the target efficiently because the PCR product is too long. Design the primers so that the PCR product is <150 bp in length.
	Design a probe that is compatible with 5.5 mM MgCl ₂ .
	For multiplex PCR, the MgCl ₂ concentration may be increased, if desired, by adding a small amount of concentrated MgCl ₂ (not provided in this kit) to the 1 × experimental reaction at the time of set up.
	The probe has a nonfunctioning fluorophore. Verify that the fluorophore functions by digesting the probe (100 nM probe in 25 µl 1 × buffer with 10 U DNase or S1 nuclease) at room temperature for 30 minutes to confirm an increase in fluorescence following digestion.
	Redesign the probe.
	The reaction is not optimized and no or insufficient product is formed. Verify formation of enough specific product by gel electrophoresis.
	For multiplex PCR of more than two targets, the master mix may need to be supplemented with additional polymerase and dNTPs (not provided in this kit).
There is increased fluorescence in control reactions without template	The reaction has been contaminated. Follow the procedures outlined in reference 12 to minimize contamination.
	Perform decontamination during amplification by including uracil-N-glycosylase (UNG) in the PCR reaction mix. See <i>Preventing Template Cross Contamination</i> in <i>General Notes</i> .
Ct reported for the no-target control (NTC) sample in experimental report is less than the total number of cycles but the curve on the amplification plot is horizontal	Variation in fluorescence intensity. Review the amplification plot and, if appropriate, adjust the threshold accordingly.

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ENDNOTES

ABI PRISM® and Primer Express® are registered trademarks of The Perkin-Elmer Corporation.

TaqMan® is a registered trademark of Roche Molecular Systems, Inc.

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at <http://www.genomics.agilent.com>. MSDS documents are not included with product shipments.

STRATAGENE

An Agilent Technologies Division

BRILLIANT QPCR MASTER MIX

Catalog #600549, #929549

QUICK-REFERENCE PROTOCOL

1. If the passive reference dye will be included in the reaction (optional), dilute 1:500 (Mx4000 instrument) or 1:50 (ABI PRISM 7700 instrument). **Keep all solutions containing the reference dye protected from light.**

Note *If using a system other than the Mx4000, Mx3000P or Mx3005P instruments, the use of the reference dye may be required for optimal results.*

2. Thaw the Brilliant QPCR master mix and store on ice. Following initial thawing of the master mix, store the unused portion at 4°C.

Note *Multiple freeze-thaw cycles should be avoided.*

3. Prepare the experimental reaction by adding the following components *in order*:

Reagent Mixture

Nuclease-free PCR-grade H₂O to bring the final volume to 25 µl (including experimental DNA)

12.5 µl of 2× QRT-PCR master mix

x µl of experimental probe (optimized concentration)

x µl of upstream primer (optimized concentration)

x µl of downstream primer (optimized concentration)

0.375 µl of **diluted** reference dye (optional)

Note *A total reaction volume of 50 µl may also be used.*

4. Gently mix the reactions without creating bubbles (**bubbles interfere with fluorescence detection; do not vortex**).
5. Add x µl of experimental gDNA, cDNA, or plasmid DNA to each experimental reaction.
6. Gently mix the reactions without creating bubbles (**do not vortex**).
7. Centrifuge the reactions briefly.

8. Place the reactions in the instrument and run the appropriate PCR program below.

Two-step Cycling Protocol

Cycles	Duration of cycle	Temperature
1	10 minutes ^a	95°C
40	15–30 seconds ^b	95°C
	1.0 minute ^c	55–60°C ^d

^a An initial 10 minute incubation is required to fully activate the DNA polymerase.

^b For the Mx4000 instrument, use 30 seconds; for the Mx3000P, Mx3005P, and ABI PRISM 7700 instruments, use 15 seconds.

^c Set the temperature cycler to detect and report fluorescence during the annealing/extension step of each cycle.

^d Choose an appropriate annealing temperature for the primer set used.

Three-step Cycling Protocol

Cycles	Duration of cycle	Temperature
1	10 minutes ^a	95°C
40	30 seconds	95°C
	1.0 minute ^b	55–60°C ^c
	30 seconds	72°C

^a An initial 10 minute incubation is required to fully activate the DNA polymerase.

^b Set the temperature cycler to detect and report fluorescence during the annealing step of each cycle.

^c Choose an appropriate annealing temperature for the primer set used.